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Tumor Necrosis Factor-α Suppresses Hematopoiesis in Children With Myelodysplasia

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The term myelodysplasia (MDS) refers to a group of bone marrow failure syndromes which are relatively rare in childhood. The pathogenesis of MDS is unknown, but a variety of chromosomal, molecular, and cytochemical abnormalities have been reported. We describe a 4-month-old female with MDS who presented with severe neutropenia and refractory anemia with excess blasts (RAEB). Bone marrow progenitor cell assays showed decreased erythroid and myeloid colony formation as compared to normal marrow, and the patient's serum further diminished colony formation of both her own and control marrow. These observations suggested the presence of a soluble factor inhibitory to hematopoiesis. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of total RNA derived from the patient's bone marrow mononuclear cells revealed highly elevated tumor necrosis factor-α (TNF-α) mRNA levels. Using a similar RT-PCR profile, TNF-α mRNA levels were found to be elevated in two other children with myelodysplasia. We conclude that TNF-α is produced in large amounts by bone marrow mononuclear cells of children with MDS, and we hypothesize that TNF-α plays an important role in the pathophysiology of the ineffective hematopoiesis observed in MDS.

Key words: myelodysplasia, tumor necrosis factor-α, cytokines, hematopoiesis

INTRODUCTION

The myelodysplastic syndromes (MDS) are a group of rare acquired bone marrow failure disorders. A hallmark feature is ineffective hematopoiesis, manifested as peripheral cytopenia involving one or more cell lines in the setting of bone marrow hyperplasia [1]. MDS is more common in adults than in children, but progression to acute non-lymphoblastic leukemia (ANLL) occurs frequently in all age groups [2,3]. MDS has been classified traditionally by morphological criteria into five clinical categories: refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEBT), or chronic myelomonocytic leukemia (CMML) [4]. This classification has been modified recently by Passmore et al. [3] to include infant monosomy 7 (IMo7) and juvenile chronic myeloid leukemia (JCML), to classify MDS disorders more accurately in children.

Although the pathogenesis of MDS is incompletely understood, chromosomal, molecular, and cytochemical abnormalities have been described. Numeric or structural cytogenetic abnormalities most commonly occur in chromosomes 5, 7, and 8 [3,5,6]. Specific molecular abnormalities reported in MDS include N-*ras* oncogene mutations [7], over-expression of MDR-1 (P-glycoprotein) [8], and mutations in the p53 suppressor gene [9]. Cytokines provide stimulatory and inhibitory signals for modulating normal hematopoiesis; therefore, dysregulation of this balance could contribute to the clinical manifestations of MDS. Indeed, elevated serum levels of TNF-α [10] and abnormal synthesis of granulocyte colony-stimulating factor (G-CSF) [11] and granulocyte–monocyte colony-stimulating factor (GM-CSF) [12] have been described in some adult patients with MDS.

In this report, we describe a 4-month-old patient with MDS characterized by anemia, neutropenia, and excess blasts (RAEB). In vitro progenitor cell assays revealed that the infant's bone marrow had reduced numbers of colonies in comparison with normal bone marrow. Moreover, the patient's serum inhibited colony formation in normal bone marrow. These findings prompted a search for a soluble factor which might have inhibitory effects on hematopoiesis. A reverse transcriptase–polymerase chain reaction (RT-PCR) cytokine panel was performed on total RNA obtained from the patient's bone marrow mononuclear cells, and demonstrated highly elevated levels of TNF-α mRNA. An elevated serum TNF-α level was confirmed by enzyme-linked immunosorbent assay (ELISA).

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TABLE I. Hematological Parameters and Bone Marrow Profile for Three Children With Myelodysplasia*

Peripheral blood counts	Patient 1	Patient 2	Patient 3	Normal values
Hemoglobin (g/L)	77	66	122	120–160
MCV (fL)	84	104	108	79–86
Reticulocytes ($\times 10^9$/L)	44	37	38	50–125
Platelets ($\times 10^9$/L)	307	17	54	150–450
WBC ($\times 10^9$/L)	0.8	3.3	6.8	4.5–12.0
Neutrophils (%)	12	77	35	40–65
Bands (%)	0	1	1	0–1
Lymphocytes (%)	74	16	52	25–45
Monocytes (%)	13	1	10	1–12
Eosinophils (%)	1	5	2	0–5
Nucleated RBCs	0	22	0	0
Bone marrow profile				
Cellularity	Hyper	Hyper	Hypo	Normal
Cytogenetics	46XX	46XY	45XY,−7	46
Megaloblastic changes	Yes	Yes	Yes	None
Dyserythropoiesis	Yes	Yes	Yes	None
M:E ratio	1:1	1:1	5:1	2:1
Megakaryocytes	Normal	Normal	Hypo	Normal
Blasts (%)	20	<5	<5	<5
FAB classification	RAEB	RA	IMo7	

*MCV, mean corpuscular volume; WBC, white blood cell; hyper, hypercellularity; hypo, hypocellularity; M:E, myeloid:erythroid. FAB classification of MDS is described in the Introduction.

Similar RT-PCR analysis on archival bone marrow samples from two other pediatric MDS patients also revealed elevated TNF-α mRNA levels. Our findings demonstrate that TNF-α is overproduced in the bone marrow compartment in children with MDS, where its localized biological activity likely contributes to abnormal hematopoiesis.

CASE REPORTS

Patient 1

A 4-month-old white female was noted at birth to have dry and scaly skin, and later developed progressive alopecia, worsening erythema, and a desquamating skin rash. She required intravenous antibiotic therapy for otitis media and a groin abscess infected with *Pseudomonas* and *Citrobacter* species. Peripheral blood counts revealed severe anemia and neutropenia with an absolute neutrophil count (ANC) of approximately 100/μl (Table I). Lymphopenia was also present. Bone marrow aspiration revealed a hypercellular marrow with dyserythropoiesis, megaloblastic morphology in both myeloid and erythroid precursors, and 10–20% blasts, consistent with RAEB MDS.

When the patient was 2 months of age, treatment with G-CSF (10 μg/kg/day) briefly increased her white blood cells (WBC) from 0.8 to 2.5 $\times 10^9$/L; nevertheless, her WBC later returned to an abnormally low level (0.4 to 0.8 $\times 10^9$ cells/L) when G-CSF was discontinued. Weekly infusions of intravenous immunoglobulin and continuous antibiotic therapy cleared her infections. Bone marrow cytogenetic studies revealed a normal female karyotype (46 XX) without chromosomal abnormalities on three separate occasions over several months. ELISA measurement revealed a serum TNF-α level of 59 pg/ml (normal range 10–20 pg/ml; Specialty Labs Inc., Santa Monica, CA).

Because of persistent severe neutropenia, recurrent infections, and failure to thrive, she received a bone marrow transplantation at age 22 months from an unrelated donor. She initially developed mild cutaneous graft-versus-host disease, but demonstrated marrow engraftment by 4 weeks, and currently has normal peripheral blood counts 10 months following transplantation.

Patient 2

A 15-year-old white male was in good health until he developed fatigue, pallor, and anorexia. Peripheral blood counts revealed pancytopenia and bone marrow aspiration revealed hypercellularity, dyserythropoiesis, and megaloblastic changes, consistent with refractory anemia (RA) MDS (Table I). Five months after diagnosis, he developed acute myeloblastic leukemia, and died 2 months later.

Patient 3

A 5-year-old white male was noted to have hypospadias at birth. Prior to surgical repair at age 6 months, he was found to have thrombocytopenia and an elevated

mean corpuscular volume (MCV = 104 fL). Bone marrow aspiration revealed hypocellularity, dyserythropoiesis, and prominent megaloblastic changes. Bone marrow cytogenetics revealed an abnormal clone of cells with karyotype 45 XY, -7, leading to a diagnosis of infant monosomy 7 syndrome (IMo7) (Table I). His hematological status has been stable for 5 years with these myelodysplastic features.

MATERIALS AND METHODS

Bone Marrow Samples

Mononuclear cells from patient 1 were purified from heparinized bone marrow by Ficoll-Hypaque density centrifugation. Cells were then used immediately in colony assay experiments or lysed in GIT buffer (4.0 M guanidine isothiocyanate and 0.5% Sarkosyl, International Biotechnologies, Inc., New Haven, CT) as previously described [13]. Total RNA was pelleted by centrifugation for 20 hr at 36,000 rpm through a 5.7 M cesium chloride cushion (IBI). The purified bone marrow RNA was resuspended in ddH_2O and stored in liquid nitrogen. For patients 2 and 3, only archival bone marrow RNA was available for analysis.

Bone marrow was obtained from four normal donors during harvest for allogeneic bone marrow transplantation. All patient and control samples were obtained and studied in accordance with protocols approved by the Duke University Medical Center Institutional Review Board.

Serum Samples

Sera from Patient 1, as well as from two normal controls, were collected by venipuncture and stored at −80°C prior to use. Sera from patients 2 and 3 were not available for further analysis.

Colony Assays of Bone Marrow Progenitor Cells

Colony assays of primitive human hematopoietic cells were performed as described elsewhere [14]. Briefly, fresh mononuclear bone marrow cells were adjusted to a concentration of 5.0×10^6 cells/ml in RPMI 1640 (Gibco) and added to complete methylcellulose medium at a final concentration of 5.0×10^4 cells/ml (StemCell Technologies, Inc., Vancouver, Canada). Serum samples were added to the methylcellulose-buffy coat preparation at a final concentration of 10% (v/v) and incubated in duplicate in 16-mm wells (Costar Corp., Cambridge, MA) at 37°C in a humidified 5% CO_2 atmosphere. Colony growth was scored on day 16 for burst forming units–erythroid (BFU-E), colony forming units–erythroid (CFU-E), colony forming units–granulocyte/monocyte (CFU-GM), and colony forming units–megakaryocyte (CFU-Meg) from duplicate wells.

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Total RNA (1 μg) for each specimen was reverse transcribed into cDNA template using a random hexamer primer, as previously described [15]. PCR amplifications were performed in parallel for each primer pair using reagents and conditions as previously described [15]. Briefly, each reaction contained 100 ng of cDNA template, 1 μg of both primers, 100 pm of each nucleotide, and 12.5 μCi of (α-^{32}P) dCTP, and was cycled at 94°C for 30 sec, 60°C for 30 sec, then 72°C for 60 sec on a Perkin Elmer DNA Thermal Cycler. Samples were removed after 25 cycles. Five-microliter aliquots of PCR products were electrophoresed through a 6% non-denaturing polyacrylamide gel, dried for 2 hr at 50°C (Hoefer Scientific Instruments, San Francisco, CA), and exposed to X-omat film (Eastman Kodak Co, Rochester, NY) with an intensifying screen at −80°C for 1–4 days. Densitometric analyses of autoradiograms were performed using an Ultroscan Laser Densitometer (Pharmacia LKB). Densitometry was performed on autoradiograph bands of TNF-α and compared to β-actin levels to yield a numerical ratio.

Primers

Oligonucleotide primers were synthesized according to the published cDNA sequences for a panel of cytokines [16]. Primer pairs were designed to amplify the following cytokines and growth factors: TNF-α, TNF-β, γ-IFN, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-13, transforming growth factor β 1 (TGF-β1), TGF-β2, G-CSF, GM-CSF, and monocyte colony-stimulating factor (M-CSF). β-actin served as a positive control.

Statistics

Statistical analysis was performed using the Primer of Biostatistics package (McGraw-Hill, NY, NY). Sample comparisons were performed using the Student's *t*-test.

RESULTS

Progenitor Cell Assays

The results of bone marrow progenitor assays from two independent experiments are summarized in Table II. Bone marrow progenitor cells from patient 1 produced fewer total colonies in growth media when compared to cells from a normal donor ($P < .05$). Patient 1 marrow had especially diminished erythroid colony formation as compared to normal marrow ($P = .01$). The addition of serum from patient 1 had a marked inhibitory effect on the total colony formation of both the patient and the normal donor marrow ($P < .05$). In contrast, total colony formation for patient 1 and the normal donor was not significantly affected by the addition of normal serum.

TABLE II. Suppression of Hematopoiesis by a Serum Factor From Patient 1 With MDS*

Marrow	Serum	Experiment	BFU-E	CFU-E	CFU-GM	CFU-Meg	Total colonies
Patient	Media	1	4	4	2	6	16
		2	1	4	5	3	13
Normal	Media	1	3	17	4	5	29
		2	1	21	6	7	35
Patient	Patient	1	0	3	0	4	7
		2	0	3	2	3	8
Normal	Patient	1	2	7	3	6	18
		2	3	5	1	6	15
Patient	Normal	1	2	10	1	5	18
		2	3	7	2	7	19
Normal	Normal	1	5	11	5	7	28
		2	6	14	6	5	31

*Experiments were performed as described in Materials and Methods with each point run in duplicate. Results are shown for two independent experiments and demonstrate that the patient's marrow produced fewer colonies than control marrow, and the patient's serum further inhibited colony formation in both her bone marrow and normal control marrow. Normal serum did not inhibit colony formation.

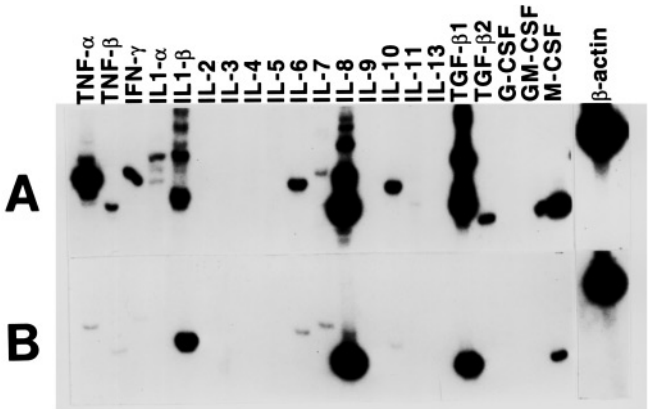

Fig. 1. TNF-α mRNA levels are highly elevated in the bone marrow mononuclear cells of patient 1. RT-PCR was performed on RNA from patient 1 bone marrow **(A)** and on normal control bone marrow **(B).** In four independent experiments, only TNF-α mRNA expression was significantly higher in the patient's bone marrow. Other cytokine mRNA levels were similar between the patient and control marrow.

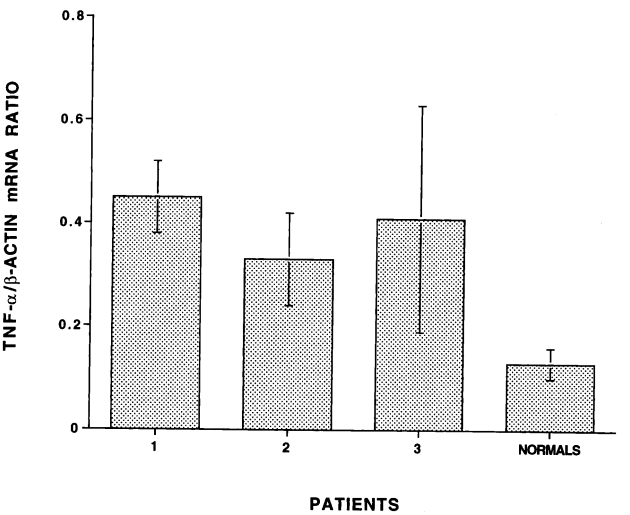

Fig. 2. TNF-α mRNA expression is elevated in childhood MDS. RT-PCR analysis was performed on three children with MDS and four normal controls, in three to four independent experiments for each sample. A numerical ratio of TNF-α/β–actin was calculated based on densitometry values for each autoradiograph, then averaged for each patient. Each patient has significantly more TNF-α expression than the normal controls ($P < .001$, $P = .017$, and $P = .041$, respectively).

RT-PCR Analysis of Bone Marrow Mononuclear Cells

A RT-PCR bone marrow cytokine profile revealed that TNF-α mRNA expression was highly elevated in patient 1 as compared to a normal control (Fig. 1). Using the TNF-α/β-actin ratio derived from densitometry of the radiograph, TNF-α mRNA levels were much higher in the bone marrow of patient 1 as compared to the control. Four independent RT-PCR experiments confirmed these results. Both bone marrow samples expressed high mRNA levels of IL-1β, IL-8, TGF-β1, and M-CSF mRNA. Slight differences in other cytokine mRNA levels were not consistently observed.

To determine whether bone marrow TNF-α mRNA levels were elevated in other children with MDS, RT-PCR cytokine analyses were performed on two archival bone marrow samples from patients with RA (patient 2) or IMo7 (patient 3). As shown in Figure 2, each of the three patients with MDS had significantly higher TNF-α/β-actin ratios when compared to normal controls ($P < .001$, $P = .017$, and $P = .041$, respectively). In contrast, other cytokine mRNA levels did not vary significantly between individual MDS patients and the control group (data not shown).

DISCUSSION

Dysregulation of hematopoietic growth factors may play an important role in the pathophysiology of the ineffective hematopoiesis observed in MDS. However,

measurement of serum cytokine levels has, to date, not identified a single cytokine which might explain all of the abnormal clinical findings of MDS [17]. Verhoef et al. [10] found elevated serum TNF-α levels, as well as elevated IL-3, IL-6, and G-CSF levels, in a subset of adults with MDS. There was a significant inverse correlation between TNF-α levels and the hemoglobin concentration, leading the authors to conclude that TNF-α might be involved in the pathogenesis of the anemia of MDS [10]. Similarly, Zoumbos et al. [18] reported elevated serum levels of both TNF-α and soluble IL-2 receptor in adults with MDS. However, neither study investigated the source of the elevated TNF-α protein levels.

The defective hematopoiesis of MDS is not well understood, but likely results from abnormal progenitor cell growth, as stromal function appears normal [19]. Using RT-PCR analysis of bone marrow from MDS patients, Hirayama and co-workers measured mRNA expression of G-CSF, stem cell factor, IL-1β, IL-6, and IL-8, and found no significant differences in cytokine mRNA expression between patients with MDS and controls [20]. Our data confirm these previous findings. In their report, however, TNF-α mRNA levels were not measured. Each of our three children with MDS had highly elevated amounts of TNF-α mRNA expression identified from bone marrow mononuclear cells. We analyzed a number of cytokines simultaneously, but only TNF-α mRNA expression was significantly different from those of normal samples of bone marrow.

TNF-α exerts a suppressive influence on bone marrow progenitor cells in vitro, with diminished numbers of both erythroid and myeloid colonies [21–23], in addition to inducing T lymphocyte activation [24] and modulating other aspects of inflammation [25]. Elevated levels of TNF-α decreased in vitro differentiation of bone marrow progenitor cells [26], possibly by inducing upregulation of Fas antigen, which is important in the regulation of programmed cell death (apoptosis) [27]. In some instances, TNF-α can induce apoptosis directly [28,29]. Raza et al. have shown that apoptosis occurs at an increased rate in the bone marrow of patients with MDS [30] and that TNF-α can be detected in MDS bone marrow biopsy specimens [31]. In addition to accelerating rates of apoptosis, TNF-α also may inhibit erythroid and myeloid differentiation directly through a specific cell membrane receptor [23]. In our studies, the serum of patient 1 inhibited bone marrow colony growth, presumably through the actions of TNF-α. Experiments with monoclonal antibodies directed against individual cytokines would be necessary, however, to determine more precisely the contribution of each cytokine to the suppression of hematopoiesis.

Finally, our data localize the source of the elevated TNF-α mRNA production to the bone marrow mononuclear cell compartment. Several different cell types can elaborate TNF-α, including activated T and B lymphocytes [32], as well as monocytes/macrophages [33]. At present, we do not know which cells are producing the TNF-α, but suggest that high local concentrations of TNF-α suppress normal hematopoiesis within the bone marrow. Dysregulation of TNF-α could be a primary event in MDS related to a specific chromosomal or molecular abnormality, or it may represent a specific but detrimental secondary response to other events within the bone marrow.

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